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Authors

Lee, Seok-Hee
Oh, Hyun-Woo
Fang, Ying
et al.

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Identification of plant compounds that disrupt the insect juvenile hormone receptor complex

Seok-Hee Lee^{a,1}, Hyun-Woo Oh^{b,1,2}, Ying Fang^a, Saes-Byeol An^a, Doo-Sang Park^c, Hyuk-Hwan Song^d, Sei-Ryang Oh^d, Soo-Young Kim^e, Seonghyun Kim^f, Namjung Kim^f, Alexander S. Raikhel^{g,2}, Yeon Ho Je^{a,h,2}, and Sang Woon Shin^{a,2}

^aDepartment of Agricultural Biotechnology, College of Agriculture and Life Science, Seoul National University, Seoul 151-742, Republic of Korea; ^bIndustrial Bio-Material Research Center, Korea Research Institute of Bioscience and Biotechnology, Daejeon 305-806, Republic of Korea; ^cMicrobiological Resource Center, Korea Research Institute of Bioscience and Biotechnology, Daejeon 305-806, Republic of Korea; ^dNatural Medicine Research Center, Korea Research Institute of Bioscience and Biotechnology, Ochang 363-883, Republic of Korea; ^eNational Institute of Biological Resources, Incheon 404-708, Republic of Korea; ^fApplied Entomology Division, National Academy of Agricultural Science, Rural Development Administration, Suwon 441-100, Republic of Korea; ^gDepartment of Entomology, University of California, Riverside, CA 92521; and ^hResearch Institute for Agriculture and Life Sciences, Seoul National University, Seoul 151-742, Republic of Korea

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Insects impact human health through vector-borne diseases and cause major economic losses by damaging crops and stored agricultural products. Insect-specific growth regulators represent attractive control agents because of their safety to the environment and humans. We identified plant compounds that serve as juvenile hormone antagonists (PJHANS). Using the yeast two-hybrid system transformed with the mosquito JH receptor as a reporter system, we demonstrate that PJHANS affect the JH receptor, methoprene-tolerant (Met), by disrupting its complex with CYCLE or FISC, formation of which is required for mediating JH action. We isolated five diterpene secondary metabolites with JH antagonist activity from two plants: *Lindera erythrocarpa* and *Solidago serotina*. They are effective in causing mortality of mosquito larvae at relatively low LD₅₀ values. Topical application of two diterpenes caused reduction in the expression of Met target genes and retardation of follicle development in mosquito ovaries. Hence, the newly discovered PJHANS may lead to development of a new class of safe and effective pesticides.

juvenile hormone | receptor | endocrine disruptor

Insects cause enormous economic damage and human suffering. Diseases transmitted by insects result in a million deaths per year (1), and insect infestation leads to annual losses of agricultural products worth billions of US dollars (2). The high toxicity of currently available insecticides presents environmental and health risks, and growing resistance and cross-resistance of insects to these existing insecticides gravely complicates the situation. Hence, there is an urgent need to develop novel effective insecticides.

Insect growth regulators (IGRs) have been devised based on insect-specific functions. Three major classes of IGRs are commercially available (3). These IGRs include juvenile hormone (JH) agonists (methoprene and pyriproxyfen), ecdysone agonists (halofenozide and tebufenozide), and chitin synthase inhibitors (buprofezine). They possess low off-target toxicity and environmental danger and have been used to control pests. JH-based IGRs are of particular interest because JH is an insect-specific hormone. JH agonists (JHAs) disrupt insect endocrine regulation, causing abnormal development and larval fatality (4). However, JHAs have limitations in the scope of their applications because they mimic and enhance the JH mode of action. Theoretically, JH antagonists (JHANS) could be used as a powerful alternative, but no effective JHANS have yet been developed.

Recent studies identified methoprene-tolerant (Met) as the JH receptor (5, 6). Met is a member of the family of basic helix-loop-helix (bHLH)-Per-Arnt-Sim (PAS) transcription factors that requires homo- or heterodimerization for DNA binding and transcriptional regulation (7). In the *Aedes aegypti* mosquito, Met forms a heterodimer with other bHLH-PAS factors, the steroid

receptor coactivator (SRC/FISC), or Cycle (CYC) in a JH-dependent manner (8, 9). JH-mediated Met-CYC binding has been quantitatively simulated using the two-hybrid yeast β -galactosidase assay in a yeast cell, Y187 (9). Thus, it is possible to test both JHAs and JHANS for their interactions with Met and its partners using this assay.

Previously, Balsam fir juvabione has been found to inhibit the metamorphosis of the linden bug, *Pyrrhocoris apterus*, suggesting that plants could use JHAs as resistance factors against insect herbivores (10, 11). However, screening of plants for JH activity revealed only a few JHAs (12). We hypothesized that JHANS, rather than JHAs, play a significant role in plant defense against insect herbivores. In this study, we observed that plants produce JHANS as secondary metabolites. Through two-hybrid yeast β -galactosidase assay screening of 1,651 plant species, we identified 53 species that exhibited putative JHAN activity based on mosquito larvicidal toxicity tests. We isolated five diterpene secondary metabolites with JHAN activity from two plants: *Lindera erythrocarpa* and *Solidago serotina*. They were effective at causing mortality of mosquito larvae at relatively low LD₅₀ values. Moreover, topical application of two of these five diterpenes

Significance

Juvenile hormone (JH) plays key roles in insect development, reproduction, and many other physiological functions. Because JH is specific to insects, it has been investigated for use as pest control. Although compounds that mimic the action of JH (JH analogues/agonists) are efficient, they have a limited scope of application. Development of potent compounds counteracting JH action (JH antagonists) would find a wider range of control applications. However, thus far, such JH antagonists have not been developed. Here, we report on the discovery of potent JH antagonists in plants, which represents an innate resistance mechanism of plants against insect herbivores. These newly discovered plant JH antagonist compounds could be used as the starting material for developing novel insecticides.

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The authors declare no conflict of interest.

¹S.-H.L. and H.-W.O. contributed equally to this work.

²To whom correspondence may be addressed. Email: hwoh@kribb.re.kr, alexander.raikhel@ucr.edu, btrus@snu.ac.kr, or shiwoong@gmail.com.

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shows a few examples. In a parallel study, we tested the effect of methoprene acid (MA) on the pyriproxyfen-mediated Met-CYC binding in the two-hybrid yeast assay. Because MA interfered with the binding in a dose-dependent manner, it was used as a positive control during screening (Figs. 1A and 2B).

To eliminate the possibility of false signals originating from anti-yeast activity of plant extracts, we tested growth inhibition of the Y2HGold yeast strain transformed with Met and CYC (see Fig. 1B for examples). The addition of several plant extracts interfered with Met-CYC binding, resulting in normal yeast growth in nonselective double dropout minimal media (DDO, -leu/-trp), whereas retarded growth was recorded in selective quadruple dropout minimal media (QDO, -leu/-trp/-his/-ade; Fig. 1B). The plant extracts that caused yeast growth retardation in both DDO and QDO media, such as extracts from *Magnolia obovata* trunks and *Dioscorea septemloba* roots, were eliminated from further screening (Fig. 1A and B). Among 83 extracts from plant species that were initially screened, 72 passed this test but 11 failed due to their inhibitory action on yeast growth. Hence, we concluded that these 72 plant extracts that directly disrupt the JH receptor complex exhibit JHAN activity.

In a third screening step, we investigated mosquito larvicidal toxicity of selected plant extracts. Ten mosquito third-instar larvae in 3 mL tap water with food mixture were treated with a 500-ppm concentration of each plant extract. Of these 72 extracts, 53 (74%) caused more than 50% larval mortality, suggesting that the larvicidal toxicity of these plant extracts is associated with PJHAN activity (Fig. 1C and Table S1). In contrast, when we tested toxicity of extracts from 110 randomly selected plant species, we found that extracts from only 22 plant species (20%) caused a similar larval mortality (Fig. 1C).

We then purified putative plant juvenile hormone antagonist (PJHAN) compounds from two of the screened plants. The extracts of the two plants were selected based on their availability for collection and the strength of their JHAN activity—specifically, *Lindera erythrocarpa* fruits and *Solidago serotina* roots, which have relatively strong and mild activities, respectively (Fig. 1A and B). Purification resulted in isolation of three diterpene compounds from *L. erythrocarpa* [LE3B (Kanakugiol), LE3E (methyl linderone), and LE3G (methyl lucidone)] and two diterpenes from *S. serotina* [SS2A (solidagoic acid A) and SS5A (Kingidiol)] (Fig. 2A) (13–17). Details of the purification process and molecular characterization are shown in Fig. S3 A–O. The molecular structures of these diterpene molecules differed from both the sesquiterpenoid structure of JHs and various terpenoid structures of JHAs (Fig. 2A).

These diterpene molecules interfered with pyriproxyfen-mediated Met-CYC binding in the β -galactosidase assay using the Y187 yeast strain in a dose-dependent manner (Fig. 2B). Binding between Met and SRC/FISC/Taiman was observed in *A. aegypti* (8), *T. castaneum* (6), and *Bombyx mori* (18). When we tested whether the plant diterpenes interfered with the pyriproxyfen-mediated binding between *A. aegypti* Met and FISC, we found that the diterpenes interfered with the JHA-dependent Met-FISC binding with very similar manner to the Met-CYC binding (Fig. 2B). These results show that the diterpenes disrupt both the hormone-mediated heterodimer of Met-CYC and that of Met-FISC. Of the five diterpenes, four also exhibited a significant larvicidal toxicity against third-instar mosquito larvae, with an LD₅₀ of less than 100 ppm at 24 h after treatment (Table 1), yet they were minimally toxic toward human embryonic kidney 293 cells, except for LE3E compound (Fig. S4).

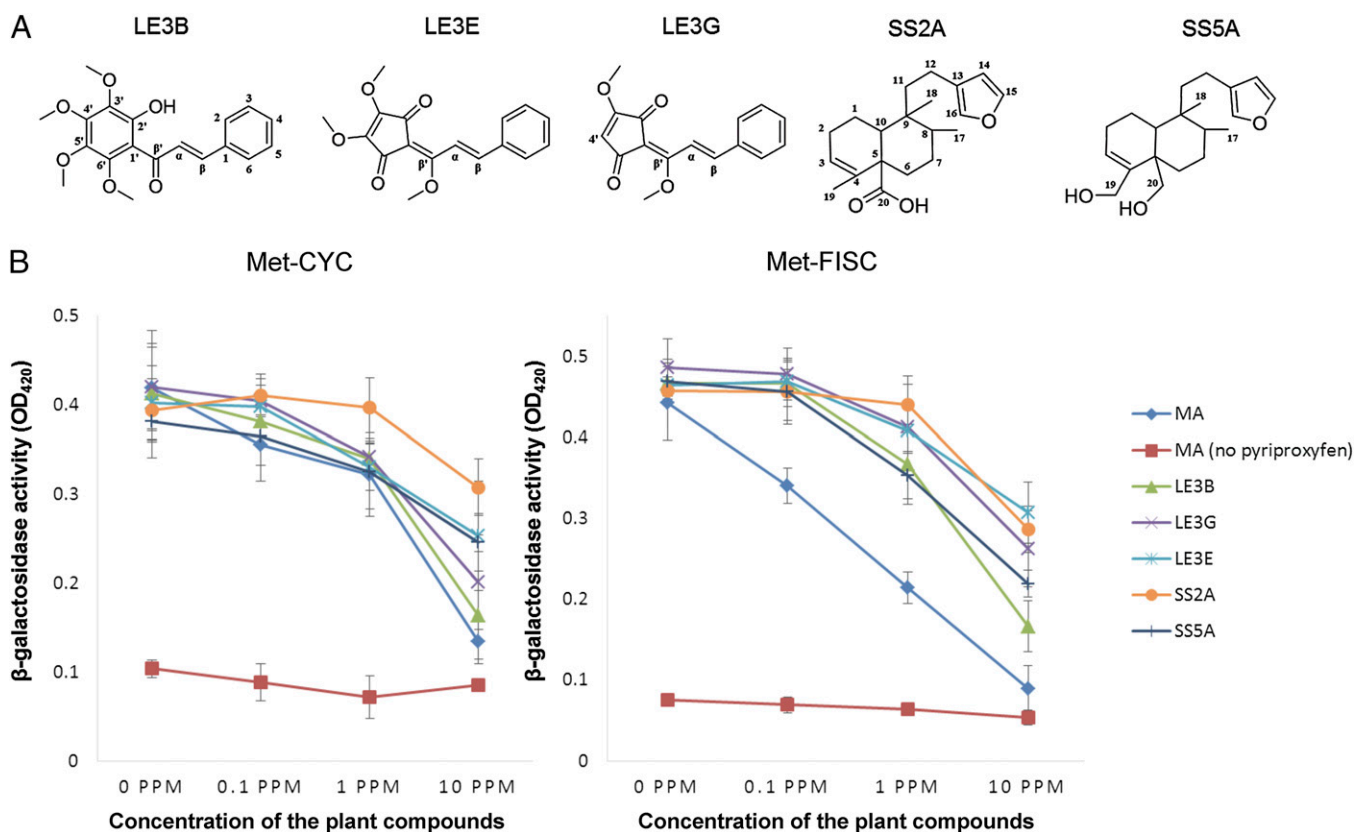


Fig. 2. PJHAN compounds and their JHAN activities. (A) Chemical structures of five PJHAN diterpene molecules isolated from *Lindera erythrocarpa* fruits and *Solidago serotina* roots identified by ¹H-NMR, ¹³C-NMR, and HRESIMS. (B) Concentration-dependent inhibition of pyriproxyfen-mediated Met-CYC or Met-FISC binding by the isolated diterpenes. To initiate the binding, 0.033 ppm of pyriproxyfen was applied into each reaction.

Table 1. Characteristics of isolated plant diterpenes

Plant extracts	Compounds	JHAN activity (10 ppm)*	LD ₅₀ at 24 h (ppm) [†]	Effect on development (0.5 µg per insect)*	Impaired gene expression (0.5 µg per insect)*
<i>Lindera erythrocarpa</i> fruits	LE3B	0.89	23	Defect of ovarian follicles ^{‡,§}	Significant reduction
	LE3E	0.62	47	ND	NS
	LE3G	0.76	31	ND	NS
<i>Solidago serotina</i> roots	SS2A	0.39	>100	ND	NS
	SS5A	0.62	85	Defect of ovarian follicles [¶]	Significant reduction
Methoprene acid		1	>100	ND	NS

ND, not detected; NS, not significant.

*Independent three replication dataset.

[†]Average of independent two replication data.

[‡]Control solvent-treated, follicle number per ovary, 102.32 ± 16.14; control follicle size, 102.31 ± 15.76.

[§]LE3B treated, follicle number per ovary, 62.25 ± 10.94 ($P = 3.7E-12$, t test); control follicle size, 66.41 ± 19.10 ($P = 7.0E-49$).

[¶]SS5A treated, follicle number per ovary, 86.33 ± 6.98 ($P = 0.029$); control follicle size, 91.27 ± 16.64 ($P = 0.054$).

Next, we conducted *in vivo* tests using the purified diterpene molecules. In the female *A. aegypti* mosquito, JH removal or Met RNA interference silencing negatively affected the gene expression of two transcriptional regulators: *Hairy* and *Kr-h1* (12). In addition, these treatments resulted in arrest of ovarian development with a significant reduction in the size of ovarian follicles (19). We applied 0.5 µg of either LE3B or SS5A, each purified in 0.2 µL acetone, topically onto the abdomens of newly emerged mosquitoes (12–24 h after eclosion). Acetone was used as a control. Two days after treatment, total RNA was prepared for real-time PCR analysis of *kr-h1* and *Hairy* gene expression. Application of two molecules, LE3B and SS5A, phenocopied the effects of JH and Met depletions. The expression of both *Hairy* and *Kr-h1* were significantly reduced (Fig. 3A). To assess whether these compounds affect development of ovarian follicles, the mosquitoes were dissected 3 d after treatment, after application of either LE3B or SS5A. These treatments resulted in the retardation of ovarian development, with significantly smaller and fewer ovarian follicles than solvent-treated mosquitoes from the control group (Fig. 3B and Table 1). These results indicate that these two diterpenes disrupt JH-dependent regulation. Mosquitoes treated with the other three diterpene molecules exhibited no significant changes in either *Hairy* or *Kr-h1* expression or in the size of ovarian follicles.

We also tested the effect of LE3B on mosquito development through longer exposure to second-instar mosquito larvae. We treated them with a sublethal concentration of LE3B (10 ppm), causing 25% (15/20) to 50% (10/20) death overnight (Table S2). In the control group, 90–95% of DMSO-treated larvae survived the night (Table S2). All of the surviving larvae in both the LE3B- or DMSO-treated samples were developed until adult emergence. Four days after emergence, female mosquitoes were dissected to observe ovary development (Fig. S5). We observed that the treatment caused neither morphological disturbance nor lethality through premature or precocious metamorphosis. Instead, we observed early adult emergence (Table S2) and a corresponding defect on ovary development in LE3B-treated mosquitoes (Fig. S5). This observation indicates that the forced early metamorphosis triggered by the plant compound may cause defects in ovary development.

Only LE3B and SS5A among five diterpenes exhibited both *in vivo* antagonistic activity against JH/JHA on Met target gene expression and ovary development. Although the other three diterpenes and methoprene acid harbored *in vitro* antagonistic activity against JH/JHA, they did not harbor *in vivo* antagonistic activity. When we tested whether the plant diterpenes antagonize the JHIII-mediated binding between Met and CYC, we found that LE3B and SS5A interfered more specifically with the natural JH rather than other three diterpenes (Fig. 4). We also

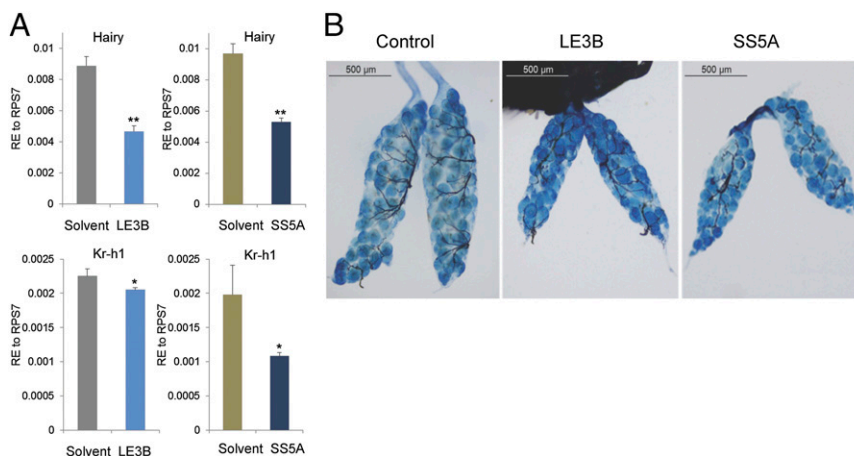


Fig. 3. JHAN activities of LE3B and SS5A in adult female mosquitoes. (A) The impaired expression of JH-activated genes, *Hairy* and *Kr-h1*, 48 h after treatment of female mosquitoes with 0.5 µg of these compounds. (B) LE3B and SS5A impaired ovarian development in treated female mosquitoes. (Left) Normal development of ovarian follicles from a female mosquito treated with solvent. (Center and Right) Ovaries from a 4-d-old female mosquito after treatment with 0.5 µg LE3B or SS5A per mosquito.

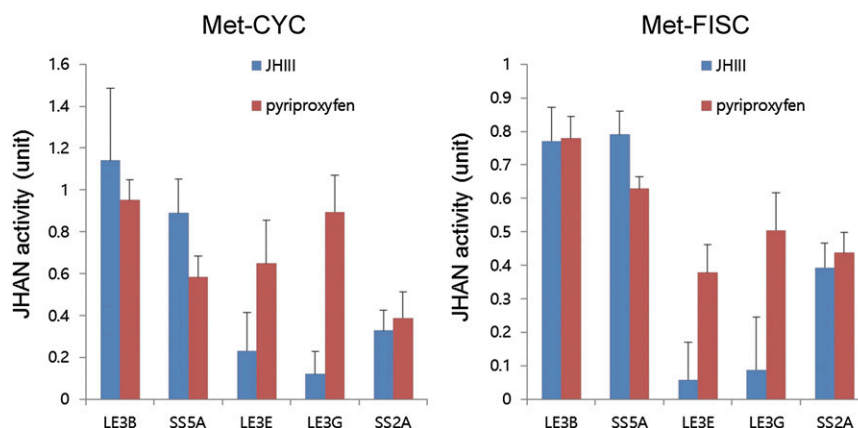


Fig. 4. Comparison between antagonistic activity against JHIII and one against pyriproxyfen by the plant diterpenes in the hormone-mediated binding of the JH receptor and the partners. The binding initiated by 1 ppm of JHIII or 33 ppb of pyriproxyfen was interfered with 10 ppm of each plant diterpene.

observed a similar result when we tested interference by the plant compounds in the JHIII-mediated Met-FISC binding (Fig. 4). This result may contribute to the observed *in vivo* activity of these two diterpenes. MA did disrupt both JHIII- and pyriproxyfen-mediated binding between Met and its partners, but we could not observe any *in vivo* antagonistic activity by MA. The molecular details regarding why only LE3B and S55A had *in vivo* antagonistic activity against natural JH and the association of JHAN activity of plant extracts and diterpenes with larvicidal toxicity need to be addressed in future studies.

Overall, we found that 53 plant species from 28 families exhibited JHAN activity and larvicidal toxicity against third-instar mosquito larvae (Table S2). Of these samples, four plant families were significantly overrepresented (hypergeometrical distribution, $P < 0.01$): (i) *Araliaceae* and (ii) *Lauraceae* from *Archichlamydeae* and (iii) *Cupressaceae* and (iv) *Pinaceae* from *Coniferosida* (Table S3). This result suggests that the presence of PJHAN compounds in plants is closely associated with their evolution. Plants deploy secondary metabolites as major constituents of defense against herbivores. It has been suggested that secondary metabolites may improve the producer's survival fitness by acting at specific receptors in competing organisms (20). All five purified diterpenes belong to secondary metabolites that have no known roles in plant functions. Thus, PJHAN diterpenes likely play a role in plant defense against insect herbivores.

Through using *in vitro* yeast two-hybrid screening assay and following *in vivo* results of PJHANS on marker gene expression and ovary development in the mosquito, we demonstrated that they disrupt JH regulation by interfering with the interaction between Met and CYC or FISC. The JH receptors belong to a family of bHLH-PAS transcription factors that is composed of nine phylogenetic clusters (12). Met factors from insects form a unique phylogenetic cluster within the bHLH-PAS protein family. Thus, PJHANS block the endocrine regulation of insect-specific receptors, indicating that they are likely to be environmentally safe. Our results indicate that newly discovered PJHAN compounds could be used as the starting material for development of novel insecticides.

Methods

Yeast Two-Hybrid Binding Test Using Growth Complementation and β -Galactosidase Assay. The cDNA fragments encoding the full ORF of each Met and CYC from three insects—*A. aegypti*, *C. pipiens*, and *T. castaneum*—and a partial ORF (M1-L689) of *T. castaneum* SRC were synthesized (Bioneer). Bait plasmids were constructed by introducing each insect Met cDNA into the GAL4 DNA binding domain of the vector pGBKT7 (Clontech). GAL4-AD fusion plasmids with each insect CYC and *T. castaneum* SRC were constructed as prey plasmids using the pGADT7 vector (Clontech). In addition, a partial ORF (M1-V510) of *A. aegypti* FISC was cloned into the pGADT7 vector. These prey

plasmids were transformed together with the Met bait plasmid into Y2HGold and Y187 yeast cells for the purpose of the yeast two-hybrid binding test using growth complementation and the quantitative β -galactosidase assay, respectively. For growth complementation, binding was tested in synthetic dropout (SD)-Leu/-Trp/-His/-Ade [quadruple dropouts (QDO)] agar medium. The transformed Y187 cells were incubated at 30 °C in SD-Leu/-Trp [double dropouts (DDO)] medium until the OD₆₀₀ reached 0.3–0.4 and were then harvested and suspended in twice the volume of the media. The cells were further incubated for 2 h, and then 100 μ L of the cells in the medium (OD₆₀₀ = 0.2–0.3) was distributed in 96-well plates. Corresponding concentrations of JH or JHA were added into the growth media, and the cells were incubated for a further 3 h and applied to the β -galactosidase assay using the Yeast β -galactosidase Assay kit (Thermo Scientific). The assay reaction mixtures in the 96-well plates were incubated at 24 °C for 16 h and then centrifuged. The supernatants were subjected to the OD₄₂₀ measurement.

Screening of Plant Extracts. The transformed Y187 cells with *A. aegypti* Met-CYC were grown following the protocol described in the previous section. Then, 100 μ L of the grown yeast cells (OD₆₀₀ = 0.2–0.3) was treated with 33 ppb pyriproxyfen and 100 ppm of each plant extract in 96-well plates. A positive control treated with 33 ppb pyriproxyfen and 10 ppm MA and a negative control treated with 33 ppb pyriproxyfen and control solvent (DMSO) were placed in each tested plate. The cells were incubated for a further 3 h and subjected to the quantitative β -galactosidase assay. The obtained OD₄₂₀ value for each plant extract-treated sample was converted to an arbitrary unit showing JHAN activity. The OD₄₂₀ value of the negative control was regarded as the 0 unit and one of the 10 ppm MA-treated samples as the 1 unit. A total of 147 extracts from 101 plant species were screened to determine whether they harbored JHAN activity of greater than 0.3 units. The repeated tests led to extracts of 83 plant species being selected for use in the subsequent screening step

$$A = \frac{\text{OD}_{420} \text{ Control} - \text{OD}_{420} \text{ PE}}{\text{OD}_{420} \text{ Control} - \text{OD}_{420} \text{ MA10}}$$

A represents the JHAN activity (if $A < 0$, then $A = 0$). The control was treated with 33 ppb pyriproxyfen. The PE group was treated with 33 ppb pyriproxyfen and 100 ppm of each plant extract. The MA10 group was treated with 33 ppb pyriproxyfen and 10 ppm MA.

Growth Inhibition and Anti-Yeast Activity Tests. The transformed Y2HGold (Clontech) yeast cells with *A. aegypti* Met-CYC were incubated at 30 °C in DDO (SD-Leu/-Trp) medium until the OD₆₀₀ reached 0.3–0.4 and then were harvested and suspended in twice the volume of the corresponding media. QDO medium was used for the growth inhibition test, and DDO medium was used for the anti-yeast activity test. The cells were incubated for a further 2 h, and then 200- μ L samples were treated with 33 ppb pyriproxyfen and 100 ppm of each screened plant extract in 96-well plates. Each sample was incubated at 30 °C with shaking, and the OD₆₀₀ of each sample was measured after a 3-h interval.

Extraction, Isolation, and Characterization of Plant Diterpenes. During October 2013, the fruits of *L. erythrorcarpa* were collected from Jeju Island, Korea,

whereas the roots of *S. serotina* were collected from Hantaek Botanical Garden, Korea. The dried fruits of *L. erythrorcarpa* (58.7 g) and the dried roots of *S. serotina* (32.7 g) were extracted three times with methanol (1 L) at room temperature to obtain 8.2 and 3.6 g of solid extract, respectively. Each methanol extract was subjected to silica gel column chromatography and was further purified by semipreparative HPLC (Gilson) using a Capcell Pak C18 column (Shiseido Co., Ltd.). LE3B, LE3G, and LE3E were isolated from *L. erythrorcarpa* fruit, whereas S52A and S55A were purified from *S. serotina* roots. The purified active compounds were analyzed using HRESIMS (Waters Q-TOF Premier), ¹H NMR, ¹³C NMR, distortionless enhancement by polarization transfer, heteronuclear multiple quantum coherence, correlation spectroscopy, and heteronuclear multiple bond coherence (Bruker AM500 MHz FT-NMR spectrometer). ¹H-NMR, ¹³C-NMR, high-resolution electrospray ionization mass spectrometry spectrums and ultraperformance liquid chromatography chromatograms of five compounds are shown in Fig. S3. The characterized chemical structures of the compounds are shown in Fig. 2A.

LE3B (Kanakugiol): yellow oil; UV (MeOH) λ_{max} nm 208, 314; ¹H NMR (400 MHz, CDCl₃) δ 7.91 (1H, d, J = 15.5 Hz, H- α), 7.82 (1H, d, J = 15.5 Hz, H- β), 7.62 (1H, m, H-2 and H-6), 7.40 (1H, m, H-3, H-4, H-5), 4.08 (3H, s, 2'-OCH₃), 3.87 (3H, s, 3'-OCH₃ and 5'-OCH₃), 3.84 (3H, s, 4'-OCH₃); ¹³C NMR (100 MHz, CDCl₃) 194.0 (C- β '), 155.2 (C-2'), 153.8 (C-4'), 151.2 (C-6'), 144.4 (C- β), 138.7 (C-5'), 137.5 (C-3'), 135.4 (C-1), 130.7 (C-4), 129.2 (C-3 and C-5), 128.7 (C-2 and C-6), 126.7 (C- α), 111.3 (C-1'), 62.4 (C-2'-OCH₃), 61.8 (C-5'-OCH₃), 61.6 (C-3'-OCH₃), 61.3 (C-4'-OCH₃); HRESIMS *m/z* [M+H]⁺ 345.1334, (calculated for C₁₉H₂₁O₆, 345.1338).

LE3E (methyl linderone): yellow solid; UV (MeOH) λ_{max} nm 248, 364; ¹H NMR (400 MHz, CDCl₃) δ 7.92 (1H, d, J = 15.9 Hz, H- α), 7.59 (1H, m, H-2 and H-6), 7.50 (1H, d, J = 15.9 Hz, H- β), 7.36 (1H, m, H-3, H-4, H-5), 4.174 (3H, s, 2'-OCH₃), 4.171 (3H, s, 3'-OCH₃), 4.08 (3H, s, β '-OCH₃); ¹³C NMR (100 MHz, CDCl₃) 187.2 (C-5'), 184.7 (C-2'), 165.4 (C- β '), 149.0 (C-3'), 147.8 (C-4'), 141.2 (C- α), 135.6 (C-1), 130.0 (C- β), 128.9 (C-3 and C-5), 128.3 (C-2 and C-6), 121.2 (C- α), 109.4 (C-1'), 64.3 (C- β '-OCH₃), 59.9 (C-2'-OCH₃), 59.8 (C-3'-OCH₃); HRESIMS *m/z* [M+H]⁺ 301.1104, (calculated for C₁₇H₁₇O₅, 301.1076).

LE3G (methyl lucidone): yellow solid; UV (MeOH) λ_{max} nm 248, 354; ¹H NMR (400 MHz, CDCl₃) δ 7.97 (1H, d, J = 15.6 Hz, H- α), 7.60 (1H, d, J = 15.9 Hz, H- β), 7.58 (1H, m, H-2 and H-6), 7.36 (1H, m, H-3, H-4, H-5), 5.92 (1H, s, H-4'), 4.17 (3H, s, β '-OCH₃), 3.91 (3H, s, 3'-OCH₃); ¹³C NMR (100 MHz, CDCl₃) 191.8 (C-5'), 185.6 (C-2'), 170.2 (C-3'), 169.0 (C- β '), 142.9 (C- β), 135.6 (C-1), 130.5 (C-4), 129.1 (C-3 and C-5), 128.7 (C-2 and C-6), 121.7 (C- α), 111.9 (C-4'), 109.4 (C-1'), 64.8 (C- β '-OCH₃), 58.7 (C-3'-OCH₃); HRESIMS *m/z* [M+H]⁺ 271.0990, (calculated for C₁₆H₁₅O₄, 271.0970).

S52A (solidagoic acid A): white solid; UV (MeOH) λ_{max} nm 217; ¹H NMR (400 MHz, CDCl₃) δ 7.20 (1H, t, J = 1.6 Hz, H-15), 7.04 (1H, s, H-16), 6.17 (1H, dd, J = 1.6, 0.8 Hz, H-14), 5.52 (1H, br s, H-3), 2.56 (1H, m, H-12a), 2.33 (1H, m, H-10), 2.27 (1H, m, H-6a), 2.19 (1H, m, H-12b), 2.07 (2H, m, H-2), 1.74 (2H, m, H-1), 1.59 (1H, m, H-7a, H-8), 1.40 (1H, m, H-6a, H-6b, H-11), 1.50 (3H, s, H-18), 1.29 (1H, m, H-7b), 0.96 (3H, s, H-19) 0.84 (3H, d, J = 6.4 Hz, H-17); ¹³C NMR (100 MHz, CDCl₃) 183.7 (C-20), 142.5 (C-15), 138.7 (C-16), 135.7 (C-4), 125.8 (C-13), 124.2 (C-3), 111.3 (C-14), 51.4 (C-5), 42.0 (C-10), 38.9 (C-9), 37.4 (C-8), 32.4 (C-11), 29.3 (C-6), 28.0 (C-7), 27.1 (C-19), 26.5 (C-2), 19.6 (C-1), 19.1 (C-18), 19.0 (C-12), 16.0 (C-17); HRESIMS *m/z* [M-H]⁻ 315.1989, (calculated for C₂₀H₂₇O₃, 315.1960).

S55A (Kingidiol): white solid; UV (MeOH) λ_{max} nm 202; ¹H NMR (400 MHz, CDCl₃) δ 7.32 (1H, t, J = 1.6 Hz, H-15), 7.21 (1H, d, J = 0.4 Hz, H-16), 6.28 (1H, d, J = 0.8 Hz, H-14), 5.89 (1H, t, J = 4.8 Hz, H-3), 4.21 (1H, d, J = 11.2 Hz,

H-19a), 3.91 (1H, d, J = 11.2 Hz, H-19b), 3.79 (1H, d, J = 10.6 Hz, H-20a), 3.43 (1H, d, J = 10.6 Hz, H-20b), 2.52 (1H, m, H-12a), 2.31 (1H, m, H-12b), 2.10 (2H, m, H-2), 1.85 (1H, m, H-11a), 1.83 (1H, m, H-1a), 1.81 (1H, m, H-6a), 1.73 (1H, m, H-11b), 1.69 (1H, m, H-1b), 1.62 (1H, m, H-8), 1.49 (1H, m, H-6b, H-7a), 1.35 (1H, m, H-10), 1.32 (1H, m, H-7b), 1.03 (3H, s, H-18), 0.89 (3H, d, J = 6.8 Hz, H-17); ¹³C NMR (100 MHz, CDCl₃) 142.8 (C-15), 141.4 (C-4), 138.7 (C-16), 132.7 (C-3), 126.2 (C-13), 111.3 (C-14), 72.1 (C-20), 64.9 (C-19), 43.3 (C-5), 39.3 (C-10, C-11), 38.9 (C-9), 36.9 (C-8), 27.3 (C-6), 26.9 (C-7), 25.1 (C-2), 24.9 (C-18), 18.8 (C-1), 18.3 (C-12), 15.2 (C-17); HRESIMS *m/z* [M-H+FA]⁻ 363.2184, (calculated for C₂₀H₂₉O₃•CH₂O₂, 363.2171).

Larval Toxicity Test and LD₅₀ Determination. Ten mosquito third-instar larvae in 3 mL tap water with food mixture were treated with a 500-ppm concentration of each plant extract. The numbers of dead larvae were counted 24 h after treatment. No dead larva was found in the negative controls treated with the solvent DMSO. All experiments were performed with three replicates, and the average percentage of mortality was counted. For the determination of LD₅₀ of PJHAN compounds, 10 larvae were treated with different concentrations of each compound, with three replicates, and the numbers of dead larvae were counted 24 h after treatment. The average larval mortality data from gradual concentration were subjected to probability unit analysis to calculate LC₅₀.

In Vivo Tests of PJHANS. Half-microgram samples of the diterpene compounds in 0.2 μ L acetone were topically applied onto abdomens of newly emerged mosquitoes. Acetone was used as a control. Two days after treatment, total RNA was prepared for real-time PCR analysis of kr-h1 and Hair gene expression. To assess the development of ovarian follicles, the mosquitoes were dissected 3 d after treatment. To test the prolonged effect of LE3B, 20 mosquito second-instar larvae in 10 mL tap water with food mixture were treated with a 10-ppm concentration of LE3B. The numbers of dead larvae were counted 24 h after treatment, and the survived larvae were maintained at 26 °C until adult emergence. The density of larvae was adjusted by adding more tap water with food mixture to control samples. Then, the larvae were left until adult emergence and then the ovaries of the emerged female mosquitoes were dissected 4 d after emergence.

RNA Preparation and Real-Time RT-PCR. Total RNA was prepared using TRIzol (GIBCO/BRL). Real-time RT-PCR experiments were performed following a previously described method (8).

Maintenance of Mosquitoes and Vertebrate Approval. The mosquito *A. aegypti* UGAL/Rockefeller strain was raised as described previously (21), and all animal experiments were performed with the approval of the Institutional Animal Use and Care Committee of the Korea Research Institute of Bioscience and Biotechnology.

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